Generation of Sense and Antisense Oligonucleotides for Inhibition of the Coat Protein Gene of the *Odontoglossum* Ringspot Virus in Orchid Callus Tissue

An Honors Thesis

by

Audra Lee Carroll

Thesis Advisor
Dr. Carolyn N. Vann

x Carolyn N. Vann

Ball State University

Muncie, Indiana

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Thesis Abstract

A major goal of our laboratory is to confer resistance specifically to the *Odontoglossum* Ringspot virus (ORSV; sometimes referred to as Tobacco Mosaic Virus Strain O (TMV-O)) in orchids, which may also provide cross-protection to other pathogens. The experimental design for the entire project is presented here along with the results of several preliminary experiments. Our approach involves RT-PCR amplification of the viral coat protein gene and digestion of the cDNA into oligonucleotides. These fragments will be cloned into a selectable vector (which confers herbicide resistance) in both sense and antisense orientations, coated with tungsten beads, and shot into orchid callus tissue using a makeshift biolistic gun. The callus will be selected for transformants by herbicide resistance, and analyzed to determine which oligonucleotide was received and the effect each oligonucleotide has on pathogen resistance. The viral coat protein gene was successfully amplified using RT-PCR with specific primers. This cDNA was cloned into the TA Cloning kit vector pCR 2.1, and was amplifiable by PCR using the same virus-specific primers. The oligos have been prepared using a DNase I digestion, verified by gel electrophoresis, and currently are ready to be ligated into the plasmid vector pG35*bar*B. Callus tissue is currently being cultured, and once mature, will be used in transformation experiments. The remaining steps in this project will be completed for my masters project.

Acknowledgments

There are several people who deserve recognition for their involvement in this project. I am grateful for the preliminary research done by previous masters students Chad Hutchinson and Steve Parsons, and the undergraduate work done by Aaron Nall. In addition, much thanks goes to Craig Reed for designing the biolistic gun to be used in future work. I would also like to thank Heather Schuck for providing the electron micrographs of the viruses. Finally, Thomas Hodges kindly provided the plasmid to be used in the callus transformation experiments.

Dr. Herb Saxon was especially helpful in the callus tissue culturing experiments and was always available for counsel on the project. Ms. Fresia Steiner was a life-saver on a day-to-day basis and was always willing to lend a hand. I will always be indebted to her.

I would like to thank the Biology Department as a whole for all their help in the two years I spent working on this project.

My deepest thanks to Dr. Carolyn Vann who has been my mentor, professor, and friend for the last two years. She has molded me into a lab rat that is now more confident and self-sufficient than when I came to her. I could never repay the invaluable guidence, patience, and expertise that Dr. Vann provided. I look forward to continuing this project with her.

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Abbreviations

A	Adenine
αmRNA	Antisense Messenger Ribonucleic Acid
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
C	Cytosine
cDNA	Complementary Deoxyribonucleic Acid
CP	Coat Protein
°C	Degrees Celsius
DEPC	Diethyl Pyrocarbonate
DIG	Digoxygenin
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-N Triphosphates
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
EtOH	Ethanol
G	Guanine
M	Molar
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
mM	Millimolar
M-MLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NASA	Nucleic Acid Sequence Analysis
ng	Nanogram
ORSV	*Odontoglossum* Ringspot Virus
pH	Potential of Hydrogen
Pol	Polymerase I
rATP	Ribose Adenosine Triphosphate
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
sp	Species
T	Thymine
Taq	*Thermus aquaticus*

TBE.....Tris-borate/EDTA Electrophoresis Buffer
TE.....Tris-EDTA
TMV.....Tobacco Mosaic Virus
TMV-O.....Tobacco Mosaic Virus Strain O
T_m.....Melting Temperature
t-RNA.....Transfer Ribonucleic Acid
U.....Units
UV.....Ultra Violet
w/v.....Weight in Volume
X.....Multiplied By
XS.....Hybrid

INTRODUCTION

The Wheeler Orchid Collection in Christy Woods at Ball State University is a well-known rescue station for rare and endangered species of orchids. Because orchids do not naturally grow close to one another, viral outbreaks among these plants are largely observed in greenhouse situations. The viruses are transmitted through the sap from the orchid plant, and in greenhouses this occurs frequently because of crowded conditions (Figure 1) and unsanitary gardening techniques. Some of the viruses, such as the *Odontoglossum* Ringspot virus (ORSV; also called Tobacco Mosaic Virus strain O (TMV-O)), can be very destructive to the plant, causing necrotic spots or even death (Figure 2) (Van Regenmortel and Fraenkel-Conrat 1986). As a result, the commercial value of the orchids is threatened: preservation of species with only one surviving member left is becoming more difficult, and the inherent beauty of the plant is being compromised.

The goal of this research project was to create virus-resistant orchid tissue by introducing DNA into orchid callus tissue that encodes mRNA molecules antisense to viral RNA. In theory, the two mRNAs will form a complex via specific base-pairing rules and will render the viral RNA nonfunctional because it cannot enter the ribosome and be translated into the necessary protein. We have modeled our project after the work done by Morgan et al. (1993), in which they used antisense oligonucleotides (small pieces) in a mammalian system. Our design is a shotgun approach (Figure 3) that is aimed at finding a method that will successfully inhibit the function of the viral RNA. Transgenic orchids that are

Figure 1. Photograph of Greenhouse Crowding. A picture within the Wheeler Orchid Collection at Ball State University which illustrates the crowded conditions common in greenhouses. The orchids pictured are members of the genus *Cattleya*.

Figure 1.

Figure 2. Photograph of a Diseased Orchid. This orchid is housed in the Wheeler Orchid Collection at Ball State University and is a member of the genus *Cattleya*. The dark spots represent the necrotic lesions caused by viruses, such as the *Odontoglossum* Ringspot Virus.

Figure 2.

Figure 3. Experimental Design. The experimental design of our project illustrated in flow-sheet form. The steps prior to "Ligation into pG35*bar*B" have been completed. The remaining steps will be completed in future work.

EXPERIMENTAL DESIGN

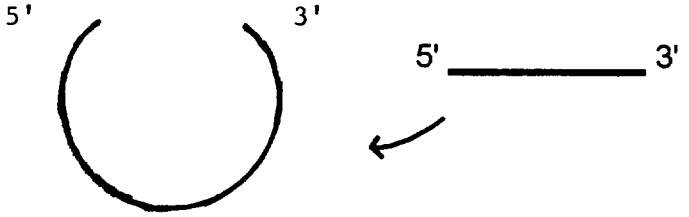
Whole ORSV (viral) RNA

5' _____ 3'
\ coat protein \

RT-PCR of Coat Protein Gene Using Virus-Specific Primers

5' _____ 3'
amplified viral cDNA

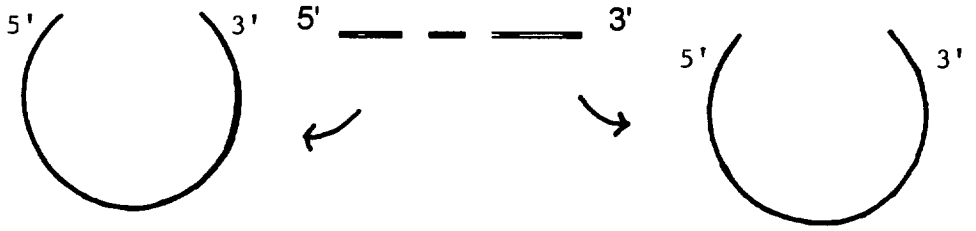
TA Cloning Kit Ligation into pCR2.1



Dnase I Digestion: Random Pool of Blunt-Ended Oligonucleotides

5' _ _ _ _ _ 3'

Ligation into pG35*bar*B (Random; Sense and Antisense)



Biolistic Bombardment of Orchid Callus Tissue with Cloned DNA

Selection of Transformants

Viral Challenge

Figure 3.

resistant to viruses, either specifically or via cross-protection, may aid in the preservation of rare species and make greenhouses safer refuges. It is also our hope that this technique will be useful in orchids because an effective transformation system for monocotyledons has not yet been developed.

The oligonucleotides constructed for this project will eventually be ligated into the plasmid pG35*bar*B, which was obtained from Thomas Hodges of Purdue University (Rathore et al., 1993). The oligos are blunt ended, and the orientation in which they will ligate into the vector will be a random process. Four different orientations are possible. The sense and antisense orientations are desired because these orientations are expected to work in a specific way. "Sense" means that the oligonucleotide is in the same orientation as the gene it was created from, and will be transcribed into a viral mRNA molecule (although it will not be full-length). Data has shown that accumulations of viral coat proteins inhibit further viral replication because the virus cannot uncoat its own coat protein to release its nucleic acid (Osbourne et al., 1989; Abel et al., 1986). We are interested in determining whether or not smaller DNA molecules of the coat protein will yield the same result. "Antisense" refers to a molecule of DNA that will encode an mRNA molecule that is complementary to the mRNA made from the DNA it was constructed from. In this case, the antisense oligonucleotides will encode mRNAs that will base pair specifically with certain regions of the viral mRNA, inhibiting the virus' replication cycle. The last two orientations that are possible code for mRNA molecules that are undesired because it is assumed that they will not be useful in

combating the virus, but due to the nature of the blunt-ended ligation these orientations are unavoidable.

The pG35*bar*B plasmid encodes the *bar* gene (see Figures 9, 10), which confers resistance to the herbicide Basta (D'Halluin et al., 1992; Rathore et al., 1993). Once the oligonucleotides are ligated into this vector, the plasmid DNA will be shot into masses of orchid callus tissue (which are currently growing) using a makeshift biolistic gun. Transformants will be selected for by treating the callus with herbicide. Any surviving callus will have received the plasmid with the resistance gene and in future work will be characterized to determine which oligonucleotide was received and to what extent it yields protection against the virus.

The biolistic gun to be used to introduce the plasmid DNA into the callus was previously constructed in our lab by Craig Reed (1993) (Figure 4). It is a mimic of the commercial biolistic gun. The plasmid DNA will be ethanol precipitated onto tungsten beads and will be injected via syringe into a stream of helium gas and into the mass of callus. Transformation efficiencies of 1% are expected (Gordan-Kamm et al., 1990) because of various difficulties in getting the DNA into the nucleus. Our lab has also achieved this efficiency using the plasmid alone. It was during this work that the conditions for particle acceleration with the makeshift gun were optimized (Parsons, 1995). This project will be continued for the completion of my masters project. Presently, the oligonucleotides are ready to be cloned into the vector plasmid. Once the callus tissue is mature, the rest of the project will follow.

Figure 4. Biolistic Gun. The makeshift biolistic gun that was constructed in our lab (Reed, 1993). Tungsten particles coated with DNA are injected via a syringe into the helium stream (Nall, 1995).

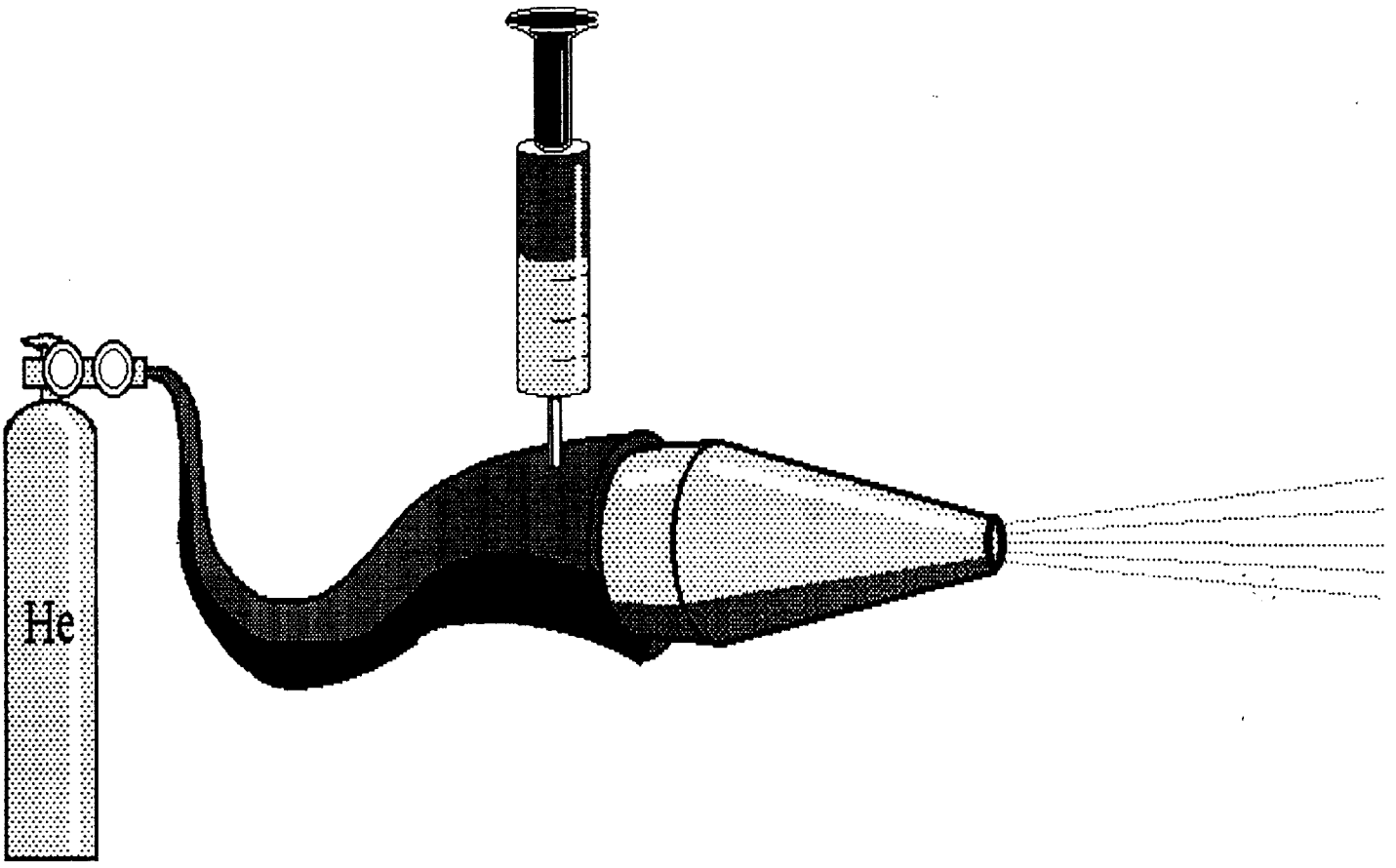


Figure 4.

Literature Review

Orchid viruses

Orchids are known to be infected with a wide variety of different viruses. However, the infections seem to be confined mostly to cultivated orchids due to the crowded conditions in greenhouses and the use of unsanitary gardening techniques (Elliott et al., 1996; Wisler et al., 1979). The only record to date of orchids infected with a virus in the wild was reported by Yao et al. (1994), in which the tomato ringspot nepovirus was found in the terrestrial orchid *Ponthieva racemosa*. Several orchid genera have been observed to exhibit viral infections (Wisler et al., 1979; Elliott et al., 1996). We have chosen to work with *Cattleya sp.* because we have successfully obtained callus tissue in the past from members of this genus and numerous species are abundant in the Wheeler Orchid Collection. In addition, this genera is susceptible to ORSV infection (Van Regenmortel and Fraenkel-Conrat 1986).

There has been some disagreement among researchers in the field about the classification of some orchid viruses. In particular are the virus(es) we are focusing on in this study, the *Odontoglossum* ringspot virus (ORSV) and tobacco mosaic virus strain O (TMV-O). It is still unclear whether these two are the same virus. In the 1950's and 1960's there were several reports of a strain of TMV (Tobacco Mosaic Virus) that infected orchids (Perez et al., 1956; Corbett, 1967; Thomson and Smirk, 1967; Kado et al., 1968). TMV and ORSV are members of the tobamovirus group of plant viruses. TMV is most notably known to infect tobacco

plants. However, recently it was reported that, other than ORSV, tobamoviruses are not serious pathogens of orchids because they do not cause systemic infection (Elliott et al., 1996). Because of the similarities in morphology of members of the tobamovirus group, it is possible that ORSV and TMV-O are the same virus because distinguishing between the virus particles is difficult (Figures 5, 6). In addition, ORSV was first identified in the orchid *Odontoglossum*. It may be that TMV-O was recognized in other orchid genera, resulting in a second name for an already existing virus. Viruses are highly mutable, and classification as a result is difficult. An analysis of the sequence of TMV-O is needed to clarify this issue, but sequence analyses of ORSV have been unclear and may complicate the issue further. Dubs and Van Regenmortel (1990) reported a sequence correction for ORSV that differed from a previous one by 31 out of 157 amino acid residues (Figure 7). For the purposes of our research, the problem is not critical because the goal is to confer resistance to orchid viruses, but the project was based on the Dubs and Van Regenmortel sequence information of the coat protein gene of ORSV using RT-PCR of TMV-O purchased from the American Type Culture Collection (ATCC). If they are two different viruses, it is hoped that cross-protection (discussed below) will be achieved because of the relative homologies of the coat protein in TMV and ORSV.

Figure 5. Electron micrograph of the *Odontoglossum* Ringspot Virus. Final magnification is X240,000 (courtesy of Heather Schuck, masters student at Ball State University).

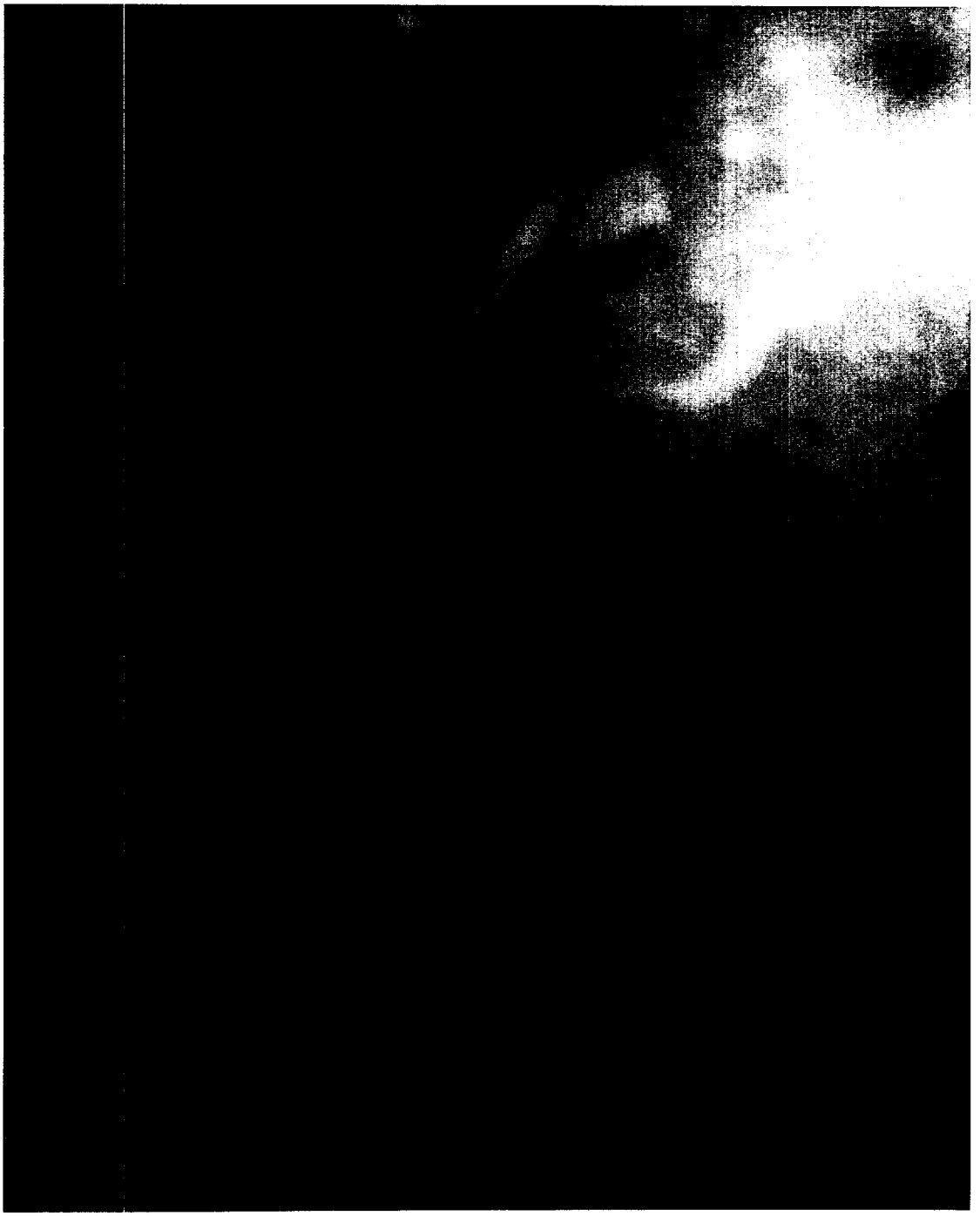


Figure 5.

Figure 6. Electron micrograph of the Tobacco Mosaic Virus Strain O. Final magnification is X240,000 (courtesy of Heather Schuck, masters student at Ball State University).

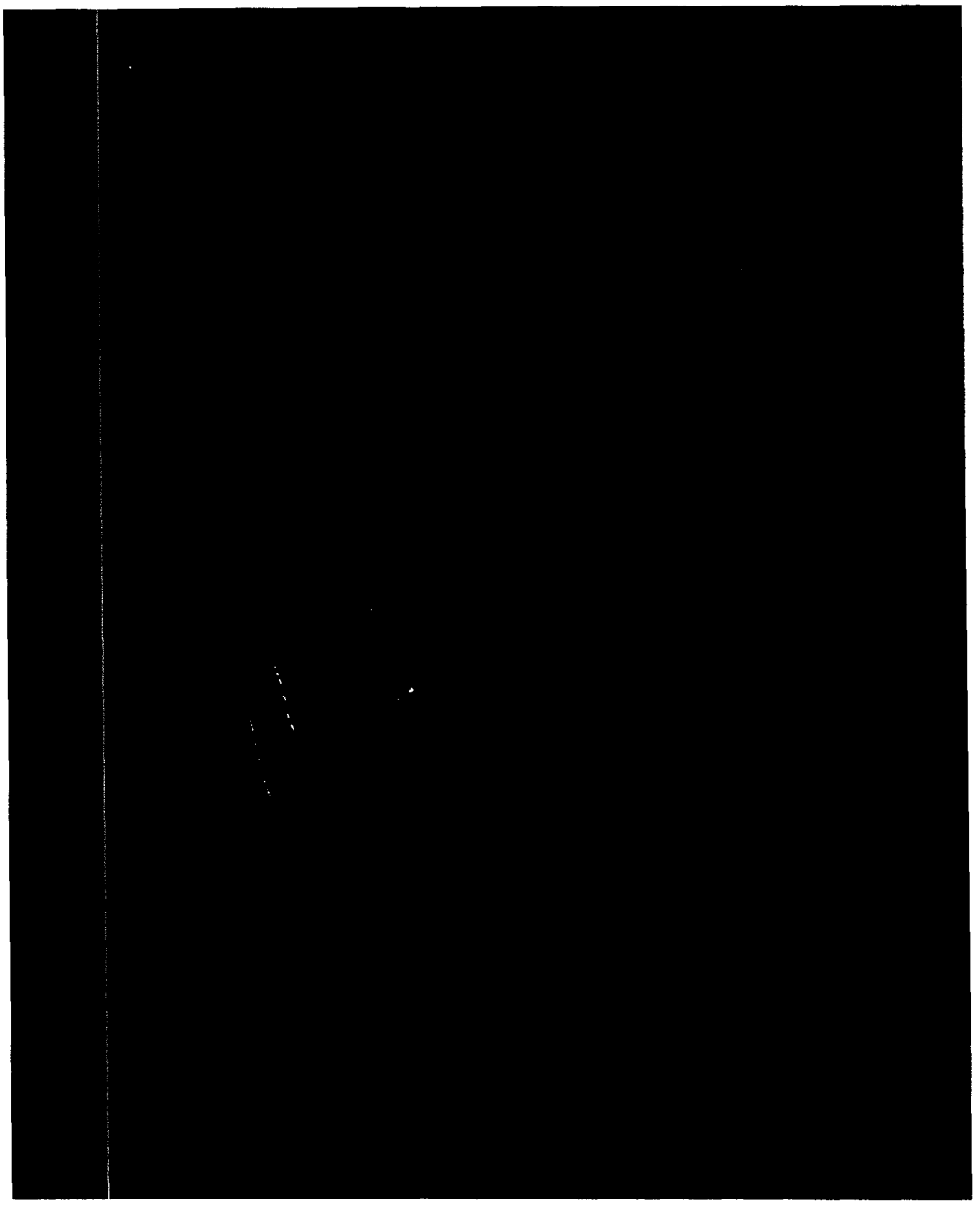


Figure 6.

Figure 7. *Odontoglossum* Ringspot Virus coat protein RNA and amino acid sequences (Dubs and Van Regenmortel, 1990). The RNA genome sequence is given as codons with the corresponding single letter amino acid code directly below each codon. The start and stop codons are in bold type. The stop codon is marked with an "*" (Hutchinson, 1992).

2 5' ACA AUC UGA UUC GUA UUG AAU **AUG** UCU UAC ACU AUU
M S Y T I

ACA GAC CCG UCU AAG CUG GCU UAU UUA AGC UCG GCU
T D P S K L A Y L S S A

UGG GCU GAC CCC AAU UCA CUA AUC AAC CUU UGU ACC
W A D P N S L I N L C T

AAU UCU CUG GGU AAU CAG UUC CAA ACA CAA CAA GCU
N S L G N Q F Q T Q Q A

CGA ACA ACU GUU CAA CAG CAG UUU GCU GAU GUU UGG
R T T V Q Q Q F A D V W

CAG CCG GUU CCU ACU UUG GCC AGU AGG UUC CCU GCA
Q P V P T L A S R F P A

GGC GCU GGU UAC UUC AGA GAU UAU CGC UAU GAU CCU
G A G Y F R D Y R Y D P

AUA UUA GAU CCU UUA AUA ACU UUC UUA AUG GGU ACU
I L D P L I T F L M G T

UUU GAU ACU CGU AAU AGA AUA AUC GAG GUA GAA AAU
F D T R N R I I E V E N

CCG CAG AAU CCG ACA ACU ACG GAA ACA UUA GAU GCA
P Q N P T T T E T L D A

ACU CGU AGA GUU GAU GAU GCA ACU GUA GCA AUA AGA
T R R V D D A T V A I R

UCU GCA AUA AAU AAU CUA UUA AAU GAG UUA GUU AGG
S A I N N L L N E L V R

GGA ACU GGU AUG UAC AAU CAA GUC UCA UUU GAG ACG
G T G M Y N Q V S F E T

AUG UCU GGA CUU ACU UGG ACC UCU UCC **UAA** UCA UAU
M S G L T W T S S *

GAG GAA AAU AAC GUU AGU GUU GAA CUA UCC GUG GUG

CAU ACG AUA AUG CAU AGU 3'

Figure 7.

Coat protein

Previous research done by Roger Beachy and coworkers on protecting plants against viral infections has largely centered on targeting the coat protein (CP) gene of these viruses. Beachy's work has been mostly concerned with protecting tobacco plants from TMV infection. It was shown that transgenic tobacco seedlings that express heightened levels of CP were delayed in developing symptoms of viral infection, although as the levels of TMV inoculum increased, the symptoms developed quicker (Abel et al., 1986). In later reports, this lab showed that disassembly of the infecting viral nucleocapsid (a process necessary for infection because it releases the viral RNA) was inhibited in transgenic tobacco plants expressing TMV CP (Osbourne et al., 1989). The mechanism of this inhibition is proposed to be the result of blocking an event that occurs before the virus uncoats itself and moves systemically (Clark et al., 1990; Wu et al., 1990).

Another result of Beachy's lab's research was the enhanced understanding of the phenomenon of cross-protection. He has shown that transgenic tobacco plants that express CP of a potyvirus (which does not cause symptoms in tobacco), confers resistance to both potato virus Y and tobacco etch virus (which are pathogens of tobacco) (Stark and Beachy, 1989). This phenomenon was also observed in tomato plants that were infected with TMV (Nelson et al., 1988). Coat protein-mediated resistance in other plants, such as squash, cantaloupe, and papaya, has also been reported (Fitch et al., 1992; Clough and Hamm, 1995).

Antisense technology

The strategy of antisense (α) inhibition is such that a molecule of mRNA introduced into a cell is complementary in sequence to that of the target RNA. The result is the formation of a αmRNA:RNA complex, which is rendered nonfunctional because it is too bulky to enter the ribosome and be translated. This method has been reported to be successful in protecting plants against viruses (Sandler et al., 1988; Powell et al., 1989). These studies used an antisense molecule that was targeted against an entire gene. Recently it has been reported that smaller antisense molecules (oligonucleotides approximately ten base pairs in length) are more efficient at ablating their target RNA (Morgan et al., 1993; Wagner et al., 1996). These studies did not involve plant viral resistance, however. The lack of antisense inhibition is thought to be due to the complex secondary structure of the target RNA which may mean certain sequences may not be fully accessible. Smaller molecules can maneuver around the structure and can bind to their target sequences more efficiently. However, there are inherent problems with using antisense oligonucleotides. The smaller oligonucleotides have a higher probability of binding non-specifically with other mRNAs, which may have detrimental consequences for the cell. In addition, targeting one sequence with a single oligonucleotide has not effectively inhibited that sequence, and certain regions of the target sequences are more susceptible to oligonucleotide binding. The concentration of oligonucleotide used has also proven to be critical because of the cellular toxicity it may cause at high levels (Morgan et al., 1993).

In order to target the coat protein gene of TMV-O more effectively, we modeled our project after that of Morgan et al. (1993). They used antisense oligonucleotides that were randomly generated by DNase I digestion. The target was an entire gene, but the random pool of oligonucleotides were directed at various sights within that gene. The object was to test which sequence was the most susceptible to the oligonucleotide and which oligonucleotide yielded the highest degree of inhibition. This is essentially the approach taken in our project, in which the coat protein gene is the target. However, included in the shotgun approach are both sense and antisense cDNA fragments. We are also cloning these fragments to permit selection of transgenics and to facilitate continuing expression of the protective cDNA fragment. Several modifications of Morgan's work, which involved a mammalian system, was necessary because we were working with a plant system.

Materials and Methods

Callus Tissue Preparation

Initially, we tried to culture meristematic orchid tissue in order to obtain callus tissue. However this method did not work and required the use of seedlings. Dr. Herb Saxon and his assistant kindly performed the initial seed culturing for this research. Subsequent subcultures were performed as described below under the direction of Dr. Saxon (Saxon, unpublished research).

Subculturing of *Cattleya* Portia coerulea 'LAKEWOOD' XS was done by aseptically transferring the tissue into freshly prepared, sterile medium. The medium was prepared by adding 900 µl water and dissolving the entire contents of the Phytamax orchid multiplication medium packet (25.3 g/L, Sigma) and autoclaving. Once the solution had cooled, 100 ml coconut water and the entire contents of the antibiotic/antimicotic solution (100X, Sigma) was added. This solution was aliquoted into twenty 125 ml flasks (previously autoclaved with cotton plugs covered with aluminum foil) with 50 ml each. The entire contents of the old medium containing the callus to be subcultured was poured into sterile petri dishes. When there were numerous small seedlings, a sterile pipet was used to transfer small portions to fresh medium. Bigger pieces of tissue were removed using sterile forceps, rinsing once with sterile water before transferring into fresh media. For callus medium that exhibited necrosis, a wash in a 3% H_2O_2 bath was done prior to the transfer for a few seconds, then rinsed with sterile water. The flasks were replaced on a rotary shaker at 60 rpms with continuous illumination. Observation

of the callus was done periodically, and subculturing done approximately every two to three weeks (Saxon, unpublished research).

Viral Isolation

After initial attempts at isolating viral RNA from orchid tissue thought to be virally infected, it was decided that whole *Odontoglossum* Ringspot Virus and Tobacco Mosaic Virus Strain O would be purchased from ATCC. This eliminated the possibility that the orchid tissue may not have been infected, or was infected with a virus other than ORSV or TMV-O.

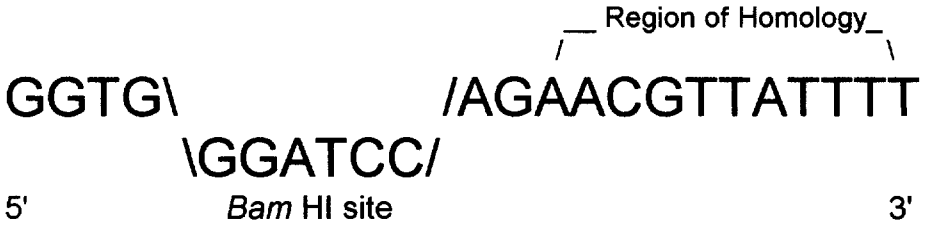
The purchased viruses had their protein coats intact, and naked RNA was obtained from TMV-O by addition of an equal volume of phenol:chloroform and a subsequent ethanol precipitation. Viral RNA concentrations were estimated using spectroscopy, and final concentrations adjusted to 2 μg for RT-PCR. Purchased TMV-O was used throughout the rest of this project to obtain viral RNA.

Primer design

The primers used were modifications of those previously designed by Aaron Null (1995). Complementary sequences of the primers were determined from the sequence of ORSV, but were named TMV-3 and TMV-4 because we used TMV-O as a template for gene amplification (see Figure 8 for primer sequences; Figure 9 shows the primers with the sequence of ORSV). TMV-3 is 23 base pairs long and its sequence in 5'-3' orientation is GGTGGGATCCAGAACGTTATTTT. TMV-4 is

Figure 8. Antisense Primer Design. The RT-PCR primers based on those designed by Nall (1995) that were used to amplify the coat protein gene of the *Odontoglossum* Ringspot Virus.

TMV-3:



TMV-4:

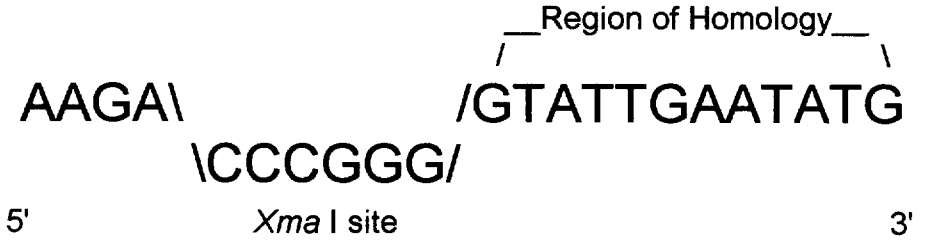


Figure 8.

Figure 9. *Odontoglossum* Ringspot Virus Coat Protein RNA and amino acid sequences with RT-PCR primers surrounding the amplified region. The RNA genome sequence is given as codons with the corresponding single letter amino acid code directly below each codon. The primers are in bold type with the restriction sites underlined. The start and stop codons are in bold, italics type (Hutchinson, 1992). The region of DNA between 5' ends of the primers was amplified, an approximately 507 base pair fragment (see Figure 12).

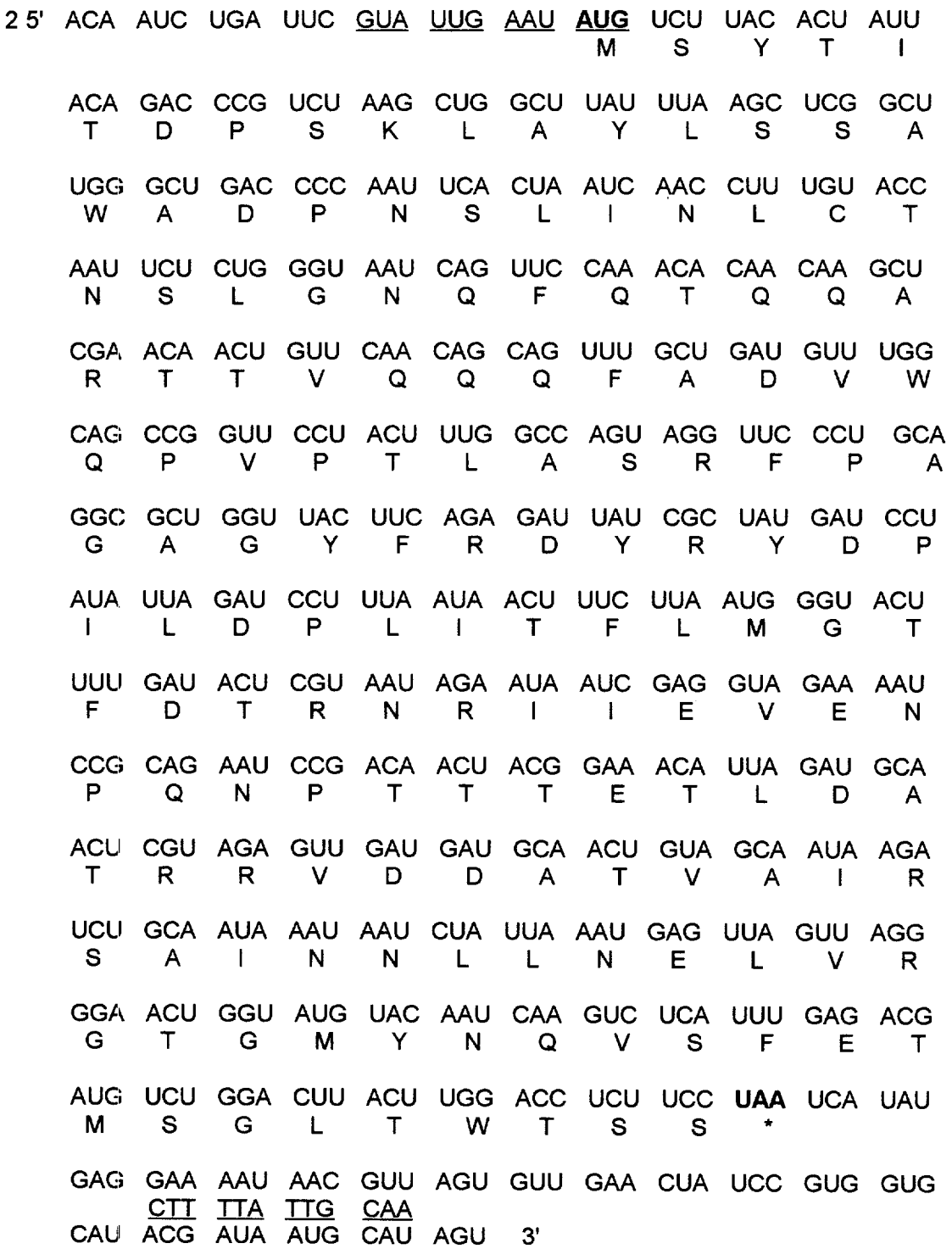

2 5' ACA AUC UGA UUC GUA UUG AAU AUG UCU UAC ACU AUU
M S Y T I
ACA GAC CCG UCU AAG CUG GCU UAU UUA AGC UCG GCU
T D P S K L A Y L S S A
UGG GCU GAC CCC AAU UCA CUA AUC AAC CUU UGU ACC
W A D P N S L I N L C T
AAU UCU CUG GGU AAU CAG UUC CAA ACA CAA CAA GCU
N S L G N Q F Q T Q Q A
CGA ACA ACU GUU CAA CAG CAG UUU GCU GAU GUU UGG
R T T V Q Q Q F A D V W
CAG CCG GUU CCU ACU UUG GCC AGU AGG UUC CCU GCA
Q P V P T L A S R F P A
GGC GCU GGU UAC UUC AGA GAU UAU CGC UAU GAU CCU
G A G Y F R D Y R Y D P
AUA UUA GAU CCU UUA AUA ACU UUC UUA AUG GGU ACU
I L D P L I T F L M G T
UUU GAU ACU CGU AAU AGA AUA AUC GAG GUA GAA AAU
F D T R N R I I E V E N
CCG CAG AAU CCG ACA ACU ACG GAA ACA UUA GAU GCA
P Q N P T T T E T L D A
ACU CGU AGA GUU GAU GAU GCA ACU GUA GCA AUA AGA
T R R V D D A T V A I R
UCU GCA AUA AAU AAU CUA UUA AAU GAG UUA GUU AGG
S A I N N L L N E L V R
GGA ACU GGU AUG UAC AAU CAA GUC UCA UUU GAG ACG
G T G M Y N Q V S F E T
AUG UCU GGA CUU ACU UGG ACC UCU UCC UAA UCA UAU
M S G L T W T S S *
GAG GAA AAU AAC GUU AGU GUU GAA CUA UCC GUG GUG
CTT TTA TTG CAA
CAU ACG AUA AUG CAU AGU 3'

Figure 9.

22 base pairs long and its sequence in 5'-3' orientation is AAGACCCGGGGTATTGAATATG. Both of these primers were synthesized by Integrated DNA Technologies, Inc.

RT-PCR

In order to make a DNA copy of the viral coat protein gene, the RNA was reverse transcribed using the enzyme reverse transcriptase. Reverse transcription was performed as follows using a modification of the conditions reported by Liang et al. (1993): 2 μg RNA was incubated at 70°C for 5 minutes to relax the secondary structure of the RNA. To this, a master mix was added to yield a final concentration of 50 mM Tris(pH 8.0), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 2 mM dNTP's (each), 1 U RNAsin, and 0.5 μg 3' primer (TMV-3). Three hundred units of M-MLV-RT (Moloney Murine Leukemia virus reverse transcriptase) was added to the experimental, and DEPC (diethyl pyrocarbonate)-treated water was added to a final volume of 30 μl. To the control, DEPC-treated water alone was added. All water, solutions, and glassware used were treated with 0.1% DEPC-treated water, which is a ribonuclease inhibitor and served to protect the viral RNA (Sambrook et al. 1989).

In order to synthesize the second strand of the single-stranded cDNA molecule made by reverse transcription, the polymerase chain reaction was performed by adding additional reagents directly to the reverse transcription product using a modification of the conditions reported by Liang et al. (1993). The master mix contained 8 μl of 10X Thermophilic Buffer (500 mM KCl, 100 mM Tris-

HCl (pH 9.0 at 25°C), and 1% Triton X-100; this yielded a final concentration of 10 mM Tris (pH 9.0) and 50 mM KCl), 2.5 mM $MgCl_2$, 1 U *Taq* polymerase, 0.5 μg 5' primer (TMV-4). DEPC-treated water was added to make the final volume 80 μl (30 μl from RT and 50 μl from PCR). The thermocycling conditions consisted of 35 cycles of 94°C for 30 seconds (denaturing), 57°C for 30 seconds (primer annealing; the temperature was calculated according to the G and C content of the primer's sequence from the commonly used formula: $T_m = 80.2°C + 0.41(G+C)$), 72°C for 2 minutes (elongation), followed by a final extension at 72°C for 10 minutes using the Perkin Elmer GeneAmp PCR System 2400. To verify the RT-PCR products, gel electrophoresis was performed using a 1.2% agarose gel in 1XTBE (0.5x:45 mM Tris-borate and 1 mM EDTA) stained with 0.15% ethidium bromide. Visualization was done with the Fotodyne Incorporated UV transilluminator (model 3-3000) and photographs were made using the BioPhotonics Corporation GelPrint 2000i.

Ligation and Transformation of viral coat protein DNA into TA Cloning Kit vector

The TA cloning kit (Invitrogen) was used to ligate the coat protein PCR product into the pCR 2.1 vector so that the cloned DNA could be transformed into *Escherichia coli*. The procedure was followed as described in the kit protocol. The cells were stored in stabs and glycerol stocks as described by Sambrook et al. (1989) for future use.

The fresh PCR products did not need to be cleaned prior to ligation according to the kit instructions. Thus, both a 1:1 and a 1:3 molar ratio of

vector:PCR product (the concentration of product was estimated by agarose gel electrophoresis) were used as suggested in the kit protocol. The reaction was incubated at approximately 12-15°C overnight.

The transformation procedure was followed as described in the kit protocol as well, except for following changes: steps 14 and 16 (in which the cells were distributed on the plates with a glass spreader) were not performed. Instead, all the ingredients were added to the top agar and this was poured onto the plates to distribute the cells evenly. A negative control was used in which no plasmid DNA was added. The positive control consisted of using pUC18 DNA (provided in the kit). Finally, since the pCR2.1 vector contained both ampicillin and kanamycin resistance genes, the antibiotic used was ampicillin.

Recovery of plasmid DNA

Toothpick streaks were made on ampicillin-containing plates of the original colonies from the transformation of both suspected transformants and suspected non-transformants. Colonies were then grown in 3 ml of terrific broth and a rapid plasmid DNA preparation was performed (Holmes and Quigley, 1981), digested with *Hind* III (15 µl plasmid, 4 µl Buffer E, 1 µl enzyme; 37°C for 1.5 hours), and run on an agarose gel (prepared as before) for 1.5 hours. Sufficient plasmid yields were not recovered using this procedure. Thus, the cultures were regrown in 3 ml of terrific broth, and an INSTA-MINI-PREP (5 Prime --> 3 Prime, Inc.) was performed. Digestion was performed as before, except that Buffer B was used for *Hind* III, and

a separate digestion with *Bam* HI (Buffer E) was used for comparison. Electrophoresis was performed as before.

Probe construction

In order to show that viral DNA was cloned into the plasmid, the viral coat protein was reamplified and used as a probe in a Southern blot. RT-PCR was performed as before, except the Boehringer Mannheim DNA labeling kit was used to label the product. In the reverse transcription master mix, 5 μl of DIG labeled DNA was added. Gel electrophoresis was done as before to verify that DNA was amplified. The labeled coat protein DNA product was precipitated by addition of 1/10 volume NaOAc and 2 volumes EtOH and 10 μl 2 mg/ml (20 μg) carrier yeast t-RNA to ensure efficient precipitation. The solution was mixed and chilled at -80°C for 30 minutes. After centrifugation in a microcentrifuge (~12,400 rpm) for 5 minutes, the pellet was washed with cold 70% EtOH, dried using a speed vacuum, dissolved in 1XTE (10 mM Tris-HCl, 1 mM EDTA) and stored at -20°C until ready for use.

Southern Analysis

Following electrophoresis, a Southern analysis was performed as described in Sambrook et al. (1989). The gels were placed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes with gentle agitation. The solution was then poured off, and the gels were rinsed three times with distilled water, and soaked in neutralizing solution (3 M NaCl, 0.5 M Tris-Cl, pH 7.6) for 30 minutes.

The Southern was set up as follows: a plastic support was placed in a glass dish containing 20XSSC (3 M NaCl, 0.3 M Na citrate). A wick (a sheet of Whatman paper) was wet in this solution and laid across the support such that the ends were submerged in the solution, taking care that no bubbles were present between the paper and the support. Two pieces of nitrocellulose membrane were cut to the same size as the gels, and soaked in 2XSSC briefly. Both gels were laid on the wick (not touching each other), and a sheet of wetted nitrocellulose was put on top of each. Three sheets (per gel) of Whatman paper (also cut to the same size as the gels) were wet in 2XSSC and laid on top of the membrane. Two stacks of paper towels approximately two inches high were cut to the size of the gels and each put onto the Whatman paper. A glass plate acting as a weight was then placed on top of the stack and the whole set-up was covered with Saran Wrap to prevent evaporation and drying out. This was left overnight to allow the DNA to transfer to the membranes. When disassembled, the nitrocellulose was left on the gel until the orientation of each gel was marked and then the membranes were rinsed in 2XSSC for 5 minutes and blotted on filter paper to dry for 5 more minutes. The blots were baked for 2 hours on clean filter paper at 80°C under vacuum, and stored in sealed plastic bags at room temperature. Prehybridization was done in 20 ml of hybridization solution (5XSSC, 0.5% blocking reagent w/v (Boehringer Mannheim kit), 0.1% N-laurylsarcosine (Na salt) w/v, 0.02% SDS w/v)) that was prepared one hour in advance and dissolved at 68°C (the solution remained turbid). The plastic seal-a-meal bags were pre-sealed on 3 sides, and after addition of the solution,

sealed using a Dazey micro-seal (leaving room to re-open the bag). The bags were left for two hours at 68°C with periodic distribution. The solution was replaced with 10 ml fresh hybridization solution containing 15 μl probe (the probe was boiled for 10 minutes in a microcentrifuge tube sealed with parafilm (a hole was poked in the top with a needle for steam to escape) to denature the DNA and quick chilled on ice (to prevent reannealing) for hybridization. Care was taken not to let the blots dry when replacing with fresh solution and the bubbles were removed from the bag so the probe would be distributed evenly over the membranes and bind to any viral DNA present. Incubation was done overnight at 68°C.

A 2 liter solution of 2XSSC and 0.1% SDS was kept at 68°C, and after hybridization was used for a series of washings (~200 ml each) which were also done at 68°C for appropriate stringency. The blots were first washed 3 times (5 minutes each) with shaking at room temperature in the warmed wash solution. Next, two 15 minutes washes were performed at 68°C, followed by three 5 minute washes with shaking at room temperature. The entire wash period should not exceed one hour. The blots were rinsed in cold 2XSSC and air dried before use.

Chemiluminescence was used to detect the probed DNA using the protocol in the Boehringer Mannheim kit. The filters were washed briefly in 100 mM Tris-HCl, 150 mM NaCl (pH 7.5), and blocking was done with a solution of 0.5% blocking reagent w/v in 100 mM Tris-HCl, 150 mM NaCl (pH 7.5) for 30 minutes. The antibody-conjugate provided by Boehringer Mannheim was diluted 1:5000 (which is stable for ~12 hours at 4°C), and 20 ml of this was incubated with the

blots for 30 minutes. The unbound antibody-conjugate was removed with two washings (100 ml) of 15 minutes each with 100 mM Tris-HCl, 150 mM NaCl (pH 7.5). Finally, the blots were equilibrated in 20 ml of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5) for two minutes. The filters were placed on an acetate sheet (a transparency sheet was used), and 1-2 ml Lumi-Phos 530 (Boehringer Mannheim) was pipetted onto them. Another acetate sheet was placed directly on top of the filters to spread the solution over the surface. This was incubated for one minute, and excess Lumi-Phos was drained. The sandwich was wrapped in Saran Wrap to prevent drying, and exposed to X-ray film in a dark-room for one and a half hours prior to developing. To develop the autoradiogram, the film was soaked in developer for two minutes, rinsed in water for one minute, soaked in fixer for two minutes, and washed in water for five minutes. The film was then hung to dry.

Reamplification of cloned plasmid DNA

To further verify that viral DNA was cloned, PCR was performed using the primers specific to the viral coat protein. Approximately 3 μg plasmid DNA was used (the concentrations were measured on a spectrophotometer). The master mix contained 60 mM Tris-HCl, 2 mM dNTP's (each), 0.5 μg each primer, 50 mM KCl, and 10 mM $MgCl_2$. To the experimental sample, 1 U *Taq* Polymerase was added, but not to the control. Water was added to a final volume of 50 μl. Gel electrophoresis was performed as before.

Preparation of Oligonucleotides

In order to increase the concentration of the cDNA, RT-PCR was performed as before four times and each 80 µl product pooled. The concentration was estimated on an agarose gel, and the remaining product was GeneCleaned (BIO 101), which will clean DNA of greater than 200 base pairs (the coat protein is 477 base pairs, but the amplified fragment is approximately 507 base pairs). The cDNA was digested with DNase I in a 100 µl reaction volume containing 2 µg DNA, 50 mM Tris-HCl pH 7.5, 10 mM $MnCl_2$, 50 µg/ml BSA, 1 µl DNase I (100 U/ml). The mixture was Incubated at 37°C for 30 minutes. The reaction was stopped by boiling the mixture for 10 minutes to inactivate the enzyme. Since DNase I in the presence of $MnCl_2$ results in fragments cut approximately at the same points on each strand, the oligonucleotides may contain one or two base pair overhangs (Sambrook et al., 1989). In order to make these ends flush for blunt end cloning, a 100 µl reaction volume containing 25 µl PCR reaction eluate, 10 µl 10X Pol I buffer (0.5 M Tris pH 7.5), 0.1 M $MgCl_2$, 10 mM DTT, 0.5 mg/ml BSA, 200 µM each dNTP, rATP to 1 mM, 10 U T4 polynucleotide kinase, 10 U DNA Pol I. The volume was adjusted to 100 µl with water, and incubated at 37°C for 1 hour. The reaction was stopped with 1 µl 0.5 M EDTA (from Double GeneClean protocol).

In order to clean the oligonucleotides, the Mermaid kit (BIO 101) was used because it selects for DNA of 10 - 300 base pairs and the desired oligonucleotide length is 10 to 50 base pairs. Three volumes High Salt Binding Solution was added to the entire mixture, and 5-8 µl of resuspended Glassfog was added per 1 µg of

DNA (the concentration was estimated on an agarose gel). This was mixed and incubated at room temperature for 5-15 minutes, vortexing during the entire period because this results in significant increases in yields of the smaller sized oligonucleotides. Centrifugation was done at high speed for a few seconds to pellet the Glassfog, which binds the DNA. An optional procedure of washing the pellet with 200 µl High Salt Binding Solution, spinning for 1-2 seconds was performed. The supernatants were combined. Three hundred microliters of Ethanol Wash was added to the Glassfog pellet and vortexed for few seconds to fully resuspend the beads. This removed salts and other compounds that will inhibit enzymes. This was centrifuged briefly and the ethanol wash repeated once or twice more with the pellet. This was centrifuged for 1-2 seconds, and the remaining supernatant discarded. The pellet was dried in a speed vacuum for a few minutes to remove excess alcohol.

The DNA was eluted from the Glassfog by resuspending the pellet in a small volume water (the same amount added above for 5-8 µl per 1 µg DNA) and incubated at 45-55°C for 5 minutes. After centrifuging for 1 minute, the supernatant was transferred to a new microcentrifuge tube and eluted as before, and the two supernatants were combined.

A 4% low MW DNA Biogel (BIO 101) agarose gel was used to separate out 10-50 base pair oligonucleotides by running the cleaned oligonucleotides for 10-15 minutes at high voltage (60-70 volts). The electrophoresis buffer that was provided with the Mermaid kit was diluted from 50X to 1X with sterile water. Other buffers

would suffice, but it was recommended that TBE not be used, especially when trying to separate out very small fragments. However, 2% agarose gels made in TBE were also used to visualize the oligonucleotide pool. The desired bands (10-50 base pairs) were cut out from the gel and placed in a microcentrifuge tube. Three volumes of High Salt Binding Solution and 8 μl of resuspended resuspended Glassfog per 1 μg DNA (binding capacity is approximately 1 μg DNA/1 μg Glassfog; excess can be added to increase binding kinetics) was added and vortexed continuously for 10 minutes (the gel "melts" rapidly at room temperature, and DNA binds to Glassfog more efficiently under vigorous mixing conditions). The Mermaid kit protocol was followed as before beginning with the first centrifugation step to remove the DNA from the agarose.

Transformation of pG35*bar*B into JM101 and JM103 competent cells

In order to have a constant source of the plasmid obtained from Thomas Hodges, competent cells were prepared and subsequent transformation performed with 50 ng pG35*bar*B as described in Sambrook et al. (1989). Top agar containing 200 μl of cells was poured onto 30 ml Luria agar plates 48 μl of 25 mg/ml ampicillin and 20 mM $MgSO_4$. Colonies were visible on the experimental plate after 17 hours. No colonies were visible on the 'no DNA' control plates. These cells were stored in Luria agar stabs and glycerol stocks as before. All were treated with ampicillin because the plasmid contains a resistance gene for this antibiotic.

Results and Discussion

Callus tissue preparation.

Initial attempts at culturing meristematic tissue were unsuccessful. Because seedlings were used instead, the initial callus will not be genetically identical, but will be "siblings" of each other. After the callus proliferates, individual clones will be made so the undifferentiated tissue will be genetically identical. The callus tissue is currently being maintained and is expected to be ready for transformation experiments soon.

Viral isolation.

Initial attempts at isolating viral RNA from orchid tissue provided by the Wheeler Orchid Collection were inconclusive because it was not clear which virus the tissue was infected with. An immunodiffusion technique known as Ouchterlony (using antibodies to both ORSV and TMV-O purchased from ATCC) was attempted without success. This led to the decision to purchase the viruses. Isolation of viral RNA was necessary so that the primers in RT-PCR would have access to the RNA for amplification. The presence of the coat protein would have inhibited this process.

Primer design.

The original objective was to create primers that would amplify the viral coat protein gene in such a way that it would be ligated into a vector in the antisense direction. Thus, the specific restrictions sites *Bam* HI and *Xma* I were incorporated into the primers for cloning into specific sites in the vector pG35*bar*B (see Figures

8, 10). However, since we decided to construct sense and antisense oligonucleotides, these specific sites were not critical. In order to ligate the oligonucleotide fragments into the plasmid, the plasmid will first be digested with the restriction enzyme *Sma* I in order to create blunt ends on the vector (Figures 10, 11).

RT-PCR.

The reaction conditions used in this process were altered several times before the viral coat protein cDNA was successfully amplified. Even after amplification was apparent, repetition yielded successively better results, which is probably due to improvements in laboratory technique. Figure 12 shows the approximately 507 base pair RT-PCR product (lane 2) that migrated slightly faster than the 603 base pair band of the ϕX174/*Hae* III marker (lane 1; see arrow) that was visualized by UV transillumination. Although the coat protein gene is 477 base pairs long from the start to the stop codon, the amplified product was expected to be approximately 30 base pairs longer because the primers flanked the regions upstream and downstream from these sites, and the band observed was approximately this size. Lane 3 is the negative control, in which no reverse transcriptase was added in the initial reaction mixture. Amplification of the coat protein gene of ORSV was not successful for unknown reasons.

Storage of the coat protein cDNA.

In order to maintain the coat protein cDNA of TMV-O for future use, we ligated it into the TA Cloning Kit vector pCR2.1 and transformed it in JM101 and

Figure 10. Restriction enzyme map of the vector plasmid pG35*bar*B (Parsons, 1995). In future work, the blunt-ended oligonucleotide fragments will be ligated into this plasmid in the SmaI site (marked with an "*") which yields blunt ends on the vector.

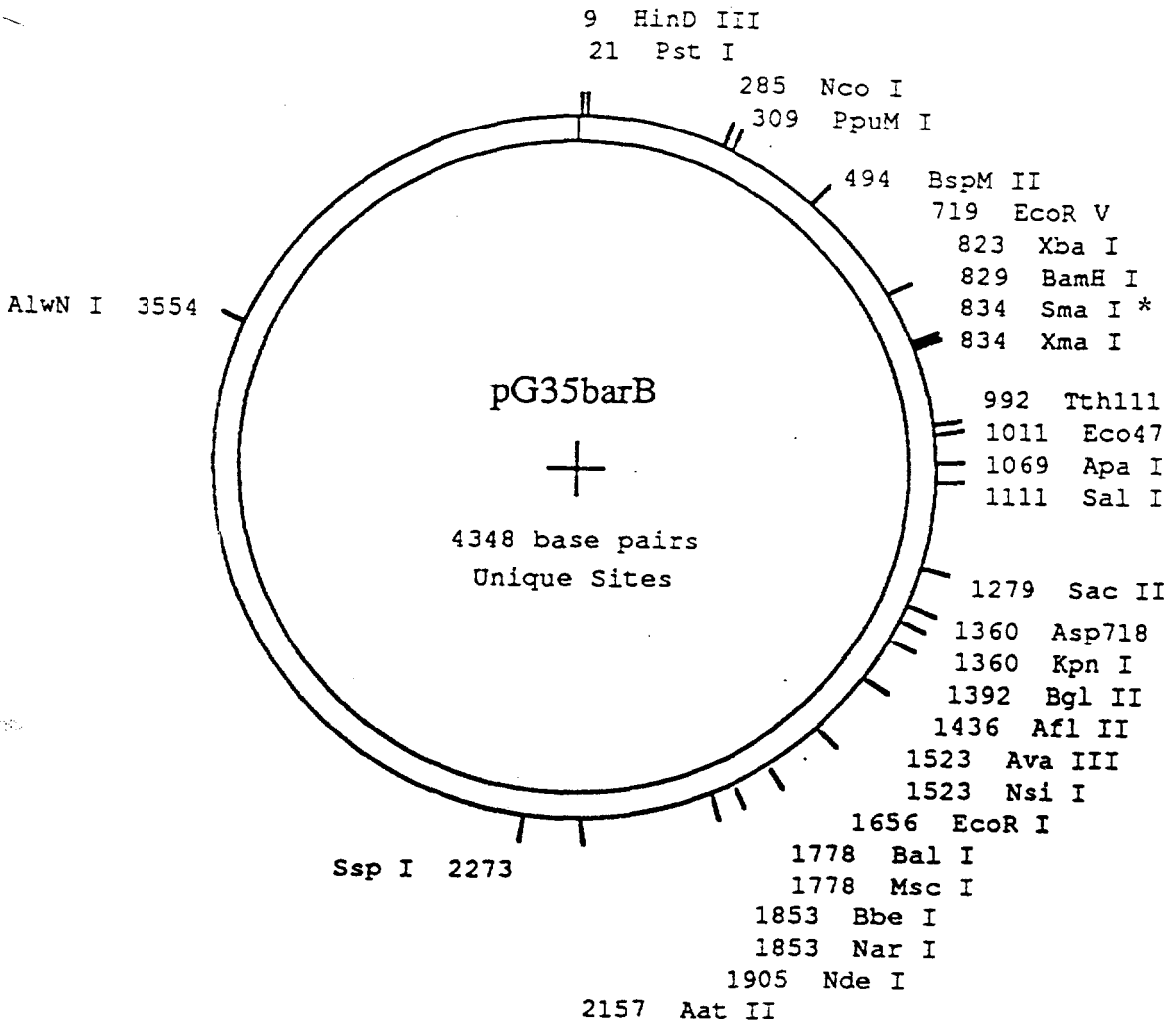

9 HinD III
21 Pst I
285 Nco I
309 PpuM I
494 BspM II
719 EcoR V
823 Xba I
829 BamH I
834 Sma I *
834 Xma I
992 Tth111
1011 Eco47
1069 Apa I
1111 Sal I
1279 Sac II
1360 Asp718
1360 Kpn I
1392 Bgl II
1436 Afl II
1523 Ava III
1523 Nsi I
1656 EcoR I
1778 Bal I
1778 Msc I
1853 Bbe I
1853 Nar I
1905 Nde I
2157 Aat II
Ssp I 2273
AlwN I 3554
pG35barB
4348 base pairs
Unique Sites

Figure 10.

Figure 11. A diagram of the vector plasmid pG35*bar*B (Rathore et al., 1993). The oligonucleotide fragments will be ligated into the region between the CaMV 35 S promoter and the *bar* gene at the *Sma* I site.

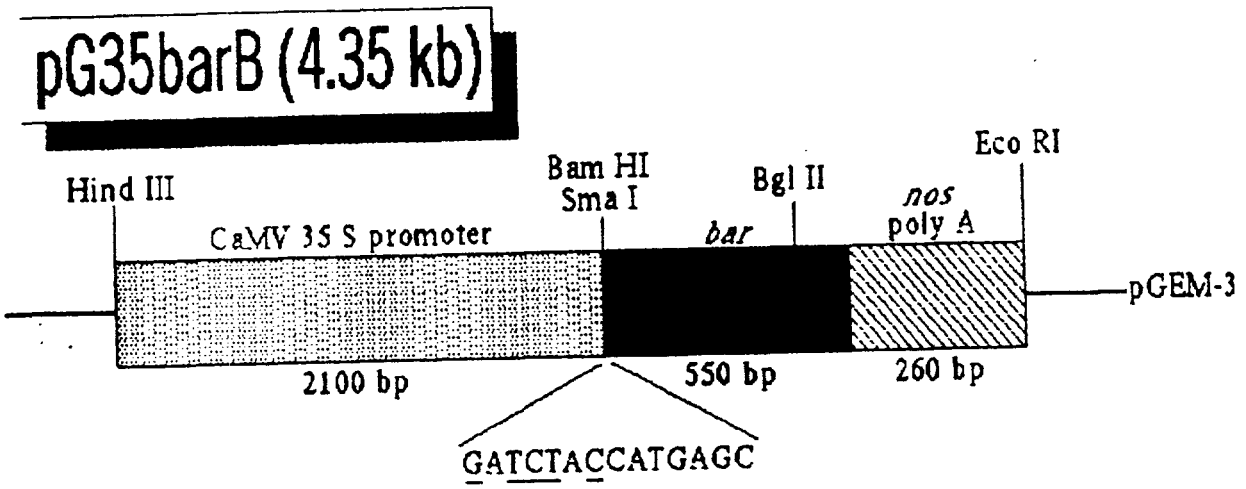

pG35barB (4.35 kb)
Hind III
Bam HI
Sma I
Bgl II
Eco RI
CaMV 35 S promoter
bar
nos
poly A
pGEM-3
2100 bp
550 bp
260 bp
GATCTACCATGAGC

Figure 11.

Figure 12. Gel electrophoresis of the RT-PCR product from amplification of the coat protein gene of the Odontoglossum Ringspot Virus using virus-specific primers. Lane 1 contained 4 μl of 0.1 μg/ml ϕX174/*Hae*III marker DNA. The band in lane 2 is 15 μl of amplified RT-PCR product from viral RNA. Lane 3 contained 15 μl of the RT-PCR product negative control. The fragment marker size is indicated on the left.

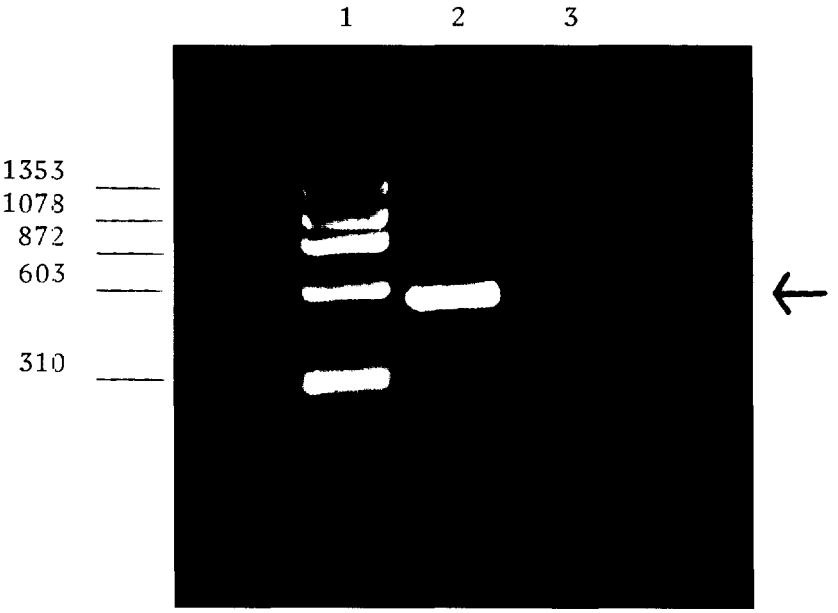


Figure 12.

JM103 *E. coli* cells. In the transformation experiment, the 1:1 molar ratio of vector:PCR product yielded more transformed colonies than the 1:3 molar ratio, although both were successful. The resulting plasmid was arbitrarily named pCRTMVO. This enabled us to perform PCR directly on the plasmid DNA so that RT-PCR of viral RNA did not need to be performed each time. To verify that the coat protein was inserted into the plasmid, two experiments were performed. First, the plasmid was digested with the restriction enzymes *Hind* III and *Bam* HI (enzymes that were determined not to cut within the coat protein DNA region using the NASA (Nucleic Acid Sequence Analysis) computer program) individually. The same was done to the pCR2.1 (containing no insert). Southern analysis of the agarose gels from the digests illustrated that viral DNA was present in pCRTMVO using the viral coat protein PCR product as the probe because binding was restricted to the plasmids containing the insert (Figures 13a, 13b). The second experiment amplified the viral fragment in the pCRTMVO plasmid using PCR and the virus-specific primers (Figure 14). The products were run on an agarose gel beside the RT-PCR coat protein product, which migrated at the same level (see arrow). The plasmid in lane 2 was discarded because the amplified fragments did not correspond to the size of the insert desired. The pCRTMVO plasmid (see carrot for corresponding banding pattern) was used to generate the coat protein for successive experiments following confirmation that it contained this gene.

Figure 13a. Restriction digests of pCRTMVO compared to uncut plasmids and uncut pCR2.1. Gel 1: lane 1 contained 4 μl of 0.1 μg/ml λ/*Hin*dIII marker DNA; lanes 2-6 contained 15 μl of the pCRTMVO plasmid digested or uncut. Lanes 2 and 6 contained plasmid cut with *Hind*III, lane 3 contained plasmid cut with *Bam*HI, and lanes 4 and 5 contained uncut plasmid. Lane 7 contained 15 μl of the linear, uncut pCR2.1 plasmid for the negative control.

Gel 2: lane I contained 4 μl of 0.1 μg/ml λ/*Hin*dIII marker DNA; lanes 2-6 contained 15 μl of the pCRTMVO plasmid digested or uncut. Lanes 2 and 5 contained plasmid cut with *Bam*HI, lanes 3 and 6 contained uncut plasmid, and lane 4 contained plasmid cut with *Hin*dIII. Lane 7 contained 15 μl of the linear, uncut pCR2.1 plasmid for the negative control. Fragment marker size is indicated between the two gels. Compare both gels with the Southern blots of these gels in Figure 13b.

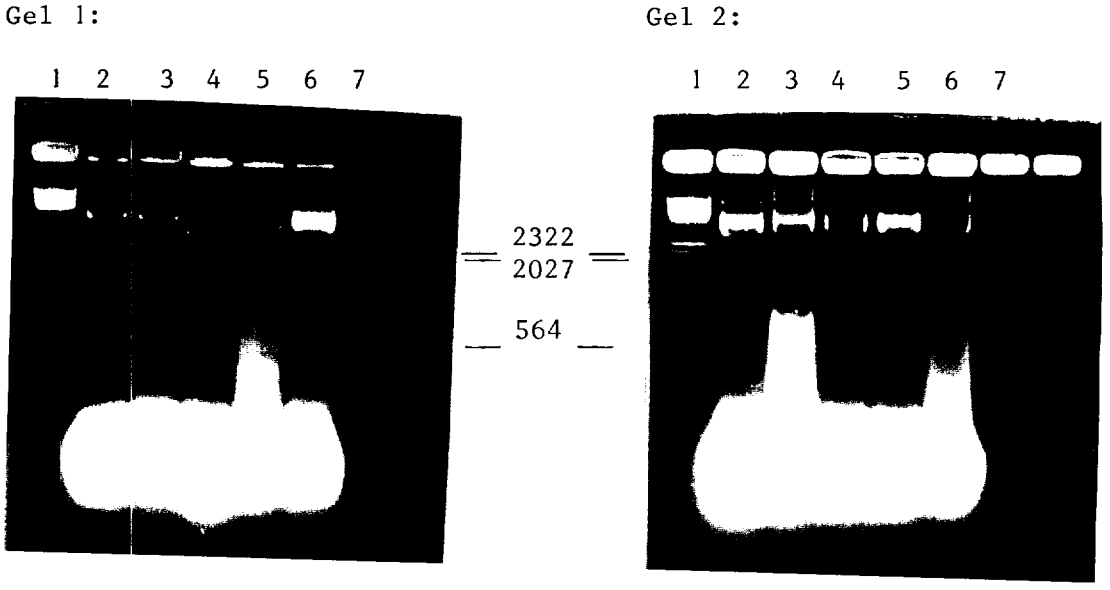


Figure 13a.

Figure 13b. Southern blots of the gels described in Figure 13a. The probe used was the amplified RT-PCR product of the viral RNA. Blots 1 and 2 correspond to Gels 1 and 2 in Figure 13a, respectively.

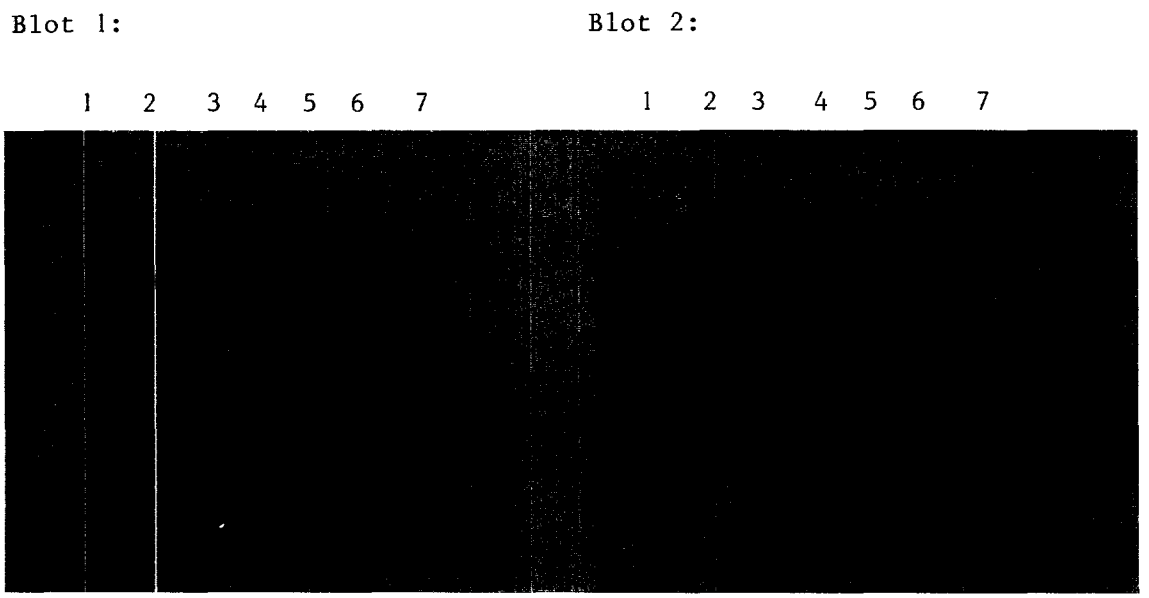


Figure 13b.

Figure 14. PCR of the pCRTMVO plasmid using virus-specific primers. Lane 1 contained 4 μl each of ϕX174/*Hae*III and λ/*Hin*dIII marker DNA. Lanes 2-7 all contained banding patterns that were not visualized in lane 8 and are associated with plasmid DNA. Lanes 2,3,5, and 7 contained 15 μl of the PCR products that had *Taq* polymerase added to the PCR reaction. Lanes 4 and 6 contained 15 ml of the PCR products that did not have *Taq* polymerase added to the reaction and served as the negative controls. Lane 8 contained 15 ml of the amplified RT-PCR product of the viral RNA and served as the positive control. Fragment marker size is indicated on the right.

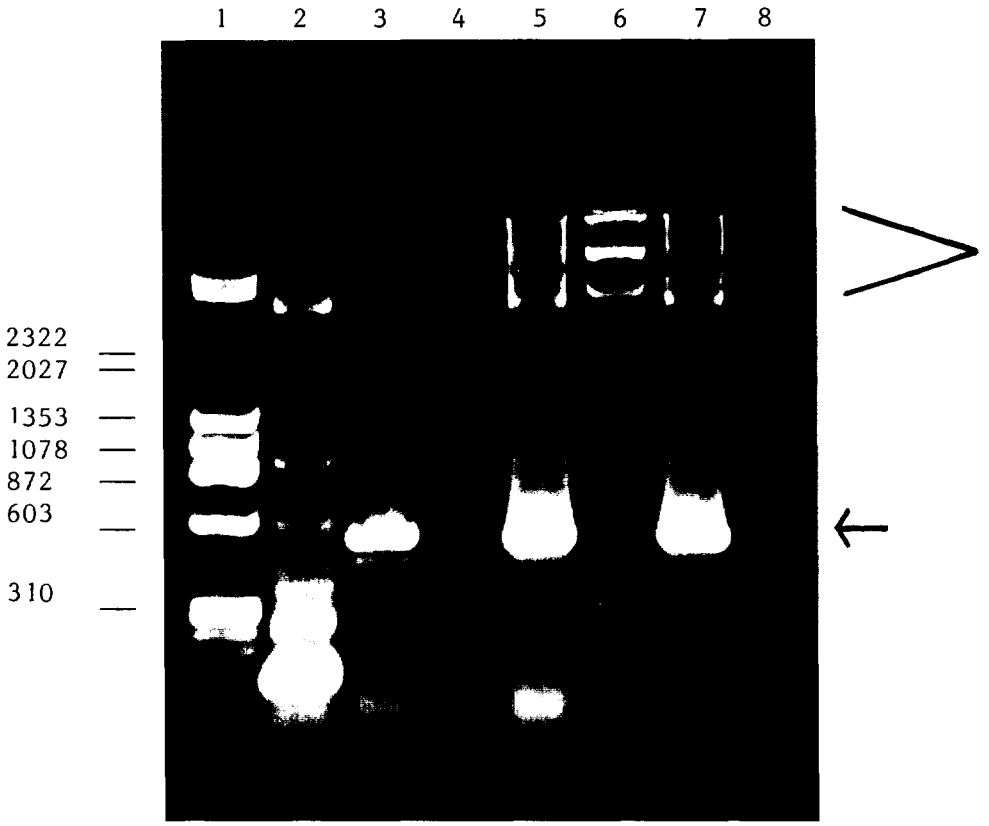


Figure 14.

Recovery of plasmid DNA.

Inconclusive results were obtained using the 'rapid method for plasmid DNA preparation' protocol (Holmes and Quigley, 1981). The INSTA-MINI-PREP kit was used in order to obtain cleaner plasmid DNA. This kit was easier to use and provided clear results. In Figures 14 and 16, the carrots are bracketing the plasmid DNA bands.

Preparation of Oligonucleotide Pool.

The coat protein DNA was digested with DNase I in order to generate a pool of short pieces of DNA (oligonucleotides) of random size. The reaction was done using manganese (Mn^{2+}) in order to generate blunt-ended fragments. DNase I cuts at approximately the same site on each strand in the presence of manganese, although 1-2 base pair overhangs may have occurred (Sambrook et al., 1989). Thus, the oligonucleotides were treated with T4 polynucleotide kinase and the Klenow enzyme to make the ends blunt-ended. Although blunt end ligations are less efficient than sticky-end ligations, this step was necessary because specific restriction sites could not be generated on each oligonucleotide in the random pool. The addition of EDTA to stop the reaction could not be done for the DNase I reaction because it would not allow the second enzymatic reaction to occur. Boiling the enzyme partially inactivates it and will allow T4 polynucleotide kinase to work (DNase I would inhibit this reaction, otherwise) (Promega Technical Representative, personal communication).

Instead of using the low MW DNA Biogel from the Mermaid kit, the pool of oligonucleotides was visualized on a 2% agarose gel in TBE buffer to determine the relative size range of the oligonucleotides (seen as a thick smear, possibly due to the presence of a high amount of salt from the two enzymatic reactions performed prior to electrophoresis). The sizes of the oligonucleotides ranged from approximately 70 to 300 base pairs in length (Figure 15, see carrot indicating the smear that represents the oligonucleotide pool). The desired length to obtain was between 10 and 50 base pairs. Further optimization of the DNase I digestion will be required to achieve this.

Isolation of pG35*bar*B from *Escherichia coli*.

The plasmid pG35*bar*B was obtained from Thomas Hodges of Purdue University. It was necessary to transform *E. coli* cells with this plasmid so that we could store this plasmid indefinitely. Both JM101 and JM103 cells were used for this purpose, and the plasmids isolated from these cells are shown migrating next to the stock plasmid in the agarose gel in Figure 16 (see carrot). The plasmid isolation from the JM101 cells was more concentrated than that from the JM103 cells.

Figure 15. Random pool of oligonucleotide fragments. Lanes 1-3 contained 15 μl of the amplified PCR product of pCRTMVO DNA. Lane 4 contained 15 μl of the negative control (see Figure 14). Lane 5 contained 4 μl of ϕX174/*Hae*III marker DNA. Lane 6 was left empty to avoid distortion of the bands because of the high salt concentration. Lanes 7 and 8 contained the entire random oligonucleotide pool generated by digestion of the viral coat protein cDNA with DNase I and treatment with T4 polynucleotide kinase and the klenow fragment. Fragment marker size is indicated on the right. The oligonucleotide fragments range between 70 and 300 base pairs in length.

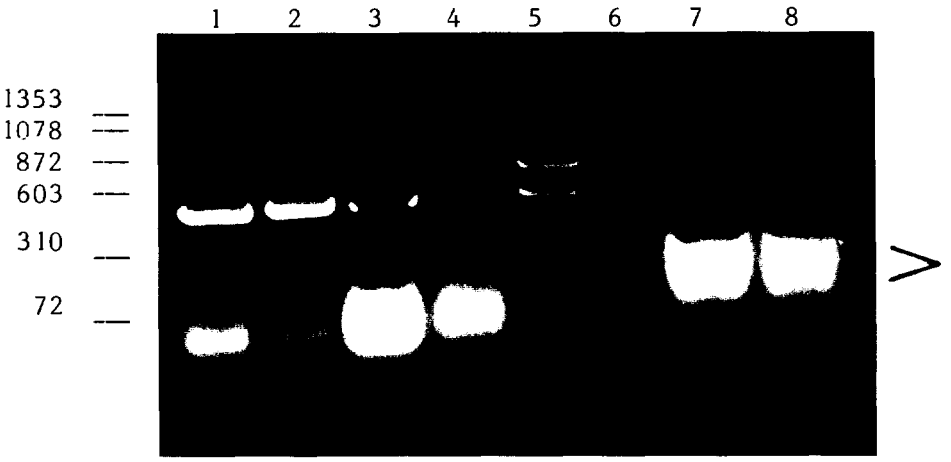


Figure 15.

Figure 16. Isolation of pG35*bar*B from *E. coli* cells. Lane 1 contained 4 μl of λ/*Hin*dIII marker DNA. Lane 2 was left empty. Lanes 3 and 4 contained 15 μl of the plasmids isolated from JM101 *E. coli* cells. Lane 5 contained 15 μl of the plasmids isolated from JM103 *E. coli* cells. Lane 6 contained 1 μl of 1mg/ml pG35barB as the positive control. Fragment marker size is indicated on the right.

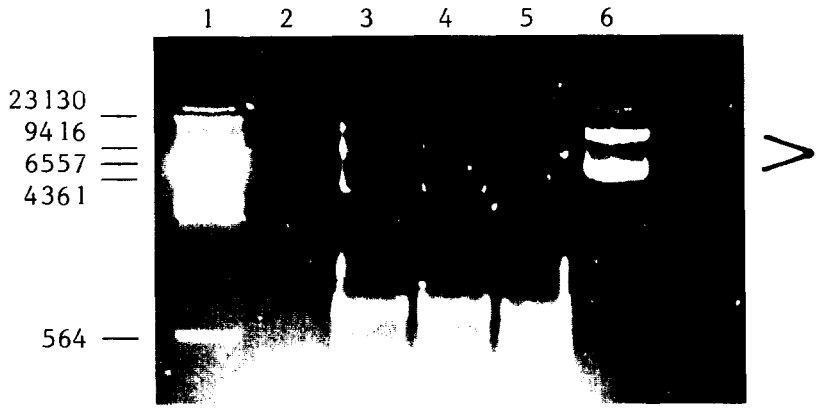


Figure 16.

Conclusions

This thesis was successful in completing some preliminary experiments ultimately targeted at conferring virus resistance in orchids. RT-PCR of purchased viral RNA was optimized, and the oligonucleotide fragments to be ligated into the vector plasmid were generated. In addition, the callus tissue is almost mature.

Based on the information gathered in this lab, the continuation of this project is promising. Future work includes ligation of the oligonucleotide fragments into the vector plasmid pG35barB, which contains a selectable marker gene that confers resistance to the herbicide Basta. The plasmid DNA containing the oligonucleotide inserts will be shot into the mature orchid callus tissue using the biolistic gun, and the transformants selected for by herbicide treatment. The transformants will be allowed to grow for a period of time before subjected to a viral challenge to determine the extent of protection the oligonucleotides conferred. The transformants will also be analyzed to determine which oligonucleotide was received, and which oligonucleotide exhibits the best protection against whole virus challenge.

The development of orchid tissue that is virus resistant will be an important advance in plant disease research. It may serve as a model for transforming other monocotyledonous plants that are agronomically important, such as wheat, corn, and rice. In addition, collections such as the Wheeler Orchid Collection will be able to combat orchid viruses from destructing rare species and the orchid's inherent aesthetic value.

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